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Expression of activated ras has been correlated with increased tumor resistance to apoptosis. We have previously demonstrated that tumors arising in MMTV-ras mice display low levels of spontaneous apoptosis and are resistant to the induction of apoptosis by chemotherapeutic agents. The goal of this project is to determine the role of two major ras effectors, raf1 and PI3K, in mediating tumor resistance to apoptosis. Transgenic mice expressing either constitutively activated or dominant negative forms of raf1 or PI3K are being created and characterized. Complementary cell culture studies are being performed to determine the effects of specific inhibitors of raf1 or PI3K on tumor cell growth and apoptosis.

To date, we have generated two lines of mice expressing activated raf1 and three lines expressing dominant-negative raf1, and high levels of transgene expression have been detected in several lines. No tumors have been observed in these mice (up to a year of age), indicating that activated raf is insufficient to mediate the full tumorigenic effect of ras. Studies underway include 1) interbreeding of these mice to various other lines of mice; 2) histological analysis and quantitation of raf signaling in mammary tissue from these mice; and construction of PI3K transgenes.

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INTRODUCTION

The Ras proteins control a wide range of cellular processes, both in normal and neoplastic cells, and a large number of effector pathways downstream of Ras are involved in mediating Ras function. One of the cellular properties of enormous clinical importance in the treatment of cancer is the relative susceptibility of tumor cells to the induction of apoptosis. In a variety of cell culture and *in vivo* model systems, activation of Ras has been shown to promote cellular resistance to apoptosis. We have extensively characterized tumors arising in MMTV-*ras* transgenic mice, and have found that these tumors exhibit very low levels of spontaneous apoptosis, and also display marked resistance to chemotherapy-induced apoptosis. Our current goal is therefore to employ both additional transgenic mouse models and cell culture studies to identify the potential role of two major Ras effector pathways (Raf and PI3K) in mediating Ras-induced apoptotic resistance. The specific aims of this proposal are to:

I. Assess the role of Raf signaling in mammary tumorigenesis in transgenic mice expressing an activated *raf* gene, and in tumor cell cultures from MMTV-*ras* tumors.
II. Similarly assess the role of PI3K in transgenic mice expressing a dominant-negative or constitutively activated PI3K gene, and in tumor cell cultures.
III. Explore the potential role of various members of the bcl-2 gene family in suppression of apoptosis in tumors and tumor cell cultures from the various classes of transgenic mice.

It is hoped that results from these studies will contribute to a better understanding of the role of Ras in tumorigenesis, tumor properties, and resistance to therapeutic treatments, and perhaps ultimately to improved strateg ies for targeting ras effector pathways in cancer therapy.

BODY

As noted in last year's summary, my laboratory moved from San Antonio, TX to Richmond VA in the spring of 2000, and our mouse colonies were moved in May, 2000. Thus, a major effort in the past year has been to rebuild the sizeable mouse colonies required for these studies from the small number of mice moved. This has progressed smoothly, and I now have new staff trained in all aspects of transgenic mouse husbandry.

During the past year, we have conducted initial characterization of the MMTV-BXB (constitutively active *raf1* mutant) and MMTV-C4B (dominant negative *raf1* mutant) lines of transgenic mice. Transgene expression has been characterized in each line of mice by Northern blot analysis of mammary gland tissue, salivary gland tissue (since the MMTV promoter also directs expression to the salivary gland), and kidney (which should not express the transgene) (see Appendix). The highest expression of C4B was detected in MMTV-C4B line 1, and so we are focusing our C4B studies on this line of mice. Mice of the one MMTV-BXB line were found to express the BXB transgene, but at relatively low levels. We have therefore initiated studies to generate additional MMTV-BXB founders, and to date have obtained one more founder, who is presently being bred to establish a line of mice.

We have also allowed a considerable number of female mice from the MMTV-BXB line 1 to age, and are monitoring them for mammary tumor development to determine whether activated *raf1* recapitulates the transforming ability of activated *ras*. To date, no tumors have been observed, suggesting that *raf1* activation alone is not responsible for the tumorigenicity exhibited by activated *ras*. However, even if *raf1* does not possess full transforming potential, it is likely to contribute to the transforming potential of *ras*. We are therefore in the process of interbreeding the MMTV-BXB mice to MMTV-*myc* and p53-/- mice to determine if activated *raf1* can cooperate with *myc* or p53-deficiency in tumorigenesis, since *ras* cooperates efficiently with both in tumorigenicity. On the flip side, we are interbreeding MMTV-C4B line 1 mice with MMTV-*ras* mice to determine

whether inactivation of the *raf* signaling pathway (by expression of the dominant negative mutant) significantly delays or alters *ras*-mediated tumorigenesis.

We are also in the process of carrying out histological characterizion of MMTV-BXB and MMTV-C4B mammary gland tissue, to determine whether alterations in *raf1* signaling perturb normal mammary gland development or involution following weaning. To do this, mammary gland tissue is being collected from female mice of each genotype (and from non-transgenic mice) at the following developmental stages: pre-puberty, post-puberty virgin, early and late pregnancy, lactating, and post-weaning. We are also initiating studies to quantitate changes in the *raf1* signaling pathway in mammary tissue from these mice, using Western blot analysis of phosphorylated MEK and MAPK.

Finally, we have obtained both constitutively active and dominant negative mutants of the PI3K gene from Dr. Paul Dent, and are starting construction of the MMTV-PI3K (mutant) transgenes.

With regard to training that has been carried out under this grant, much of the work that has been done to date has been carried out by Dr. Mark Subler, a postdoctoral fellow in my laboratory. In addition, a second-year graduate student from the Department of Human Genetics, Christina Sadler, has recently joined my laboratory, and her dissertation research will be focused on this project.

KEY RESEARCH ACCOMPLISHMENTS

- establishment of sizeable colonies of MMTV-*ras*, MMTV-C4B line 1 and MMTV-BXB line 1, and MMTV-*myc* transgenic mice, and p53-/- knock-out mice, and initiation of multiple interbreedings
- characterization of MMTV-BXB and MMTV-C4B mouse lines with regard to transgene expression and tumor development
- creation of an additional MMTV-BXB founder mouse

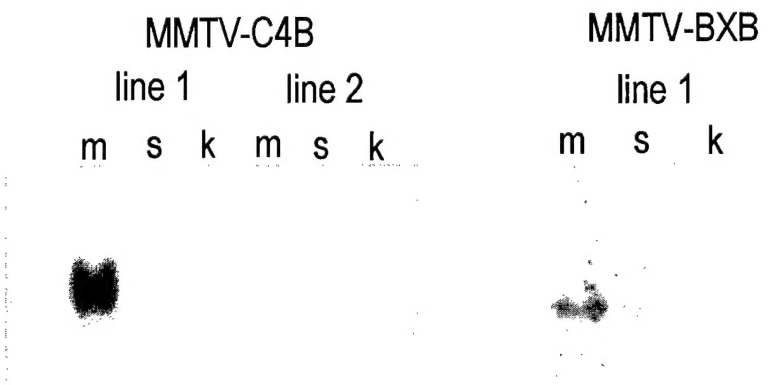
REPORTABLE OUTCOMES

- No publications have resulted from these studies yet. Publication of results from these studies will probably need to await results from the ongoing interbreeding studies.
- Two new transgenic mouse models (MMTV-BXB and MMTV-C4B) have been developed under this award. Once these mice have been well-characterized and we have published a primary description of them, they will be made readily available to other investigators who request them.

CONCLUSIONS

A preliminary conclusion from our studies to date is that activated *raf1* alone is not sufficient to induce mammary tumorigenesis in transgenic mice, and thus *raf1* signaling is not the only pathway contributing to *ras*-mediated tumorigenesis. This is essentially the result we expected, since multiple signaling pathways are thought to contribute to *ras'* tumorigenic potential. However, in the subsequent proposed studies, we hope to be able to elucidate which tumor properties (particularly apoptosis) are impacted by *raf1* and/or PI3K signaling pathways.

APPENDIX



Northern analysis of transgene expression in MMTV-C4B and MMTV-BXB transgenic mice.

Total RNA was isolated from mammary gland (m), salivary gland (s), and kidney (k) of pregnant transgenic females. Mammary-specific transgene expression was detected in MMTV-C4B line 1 and MMTV-BXB line 1 female mice.